

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problems Mailbox.**

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International BureauIDS #16  
of 09/30/97

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/72, C12P 21/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/09715</b> <b>(43) International Publication Date:</b> 24 February 2000 (24.02.00)
<b>(21) International Application Number:</b> PCT/US99/18207 <b>(22) International Filing Date:</b> 11 August 1999 (11.08.99)  <b>(30) Priority Data:</b> 60/096,326 12 August 1998 (12.08.98) US 09/198,839 24 December 1998 (24.12.98) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 09/198,839 (CIP) Filed on 24 December 1998 (24.12.98)  <b>(71) Applicant (for all designated States except US):</b> THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> KANG, Angray, S. [GB/US]; 3012 Quebrada Circle, Carlsbad, CA 92009 (US).  <b>(74) Agents:</b> NORTHRUP, Thomas, E. et al.; The Scripps Research Institute, 10550 North Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MODULATION OF POLYPEPTIDE DISPLAY ON MODIFIED FILAMENTOUS PHAGE  <b>(57) Abstract</b> <p>A modified filamentous phage that contains a gene for a wild type phage coat protein and a gene for a synthetic phage coat protein is provided. Uses of the modified phage and kits containing the phage are also provided.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Modulation of polypeptide display on modified filamentous phage

5 Funds used to support some of the studies reported herein were provided by the United States Government (Department of the Army ARL No DAAL03-92-G-0215). The United States Government may, therefore, have certain rights in the invention disclosed herein.

10 Cross Reference To Related Applications

This application is a continuation-in-part of United States Provisional Patent Application No. 60/096,326, filed August 12, 1998, the entire disclosure of which is  
15 incorporated herein by reference.

Technical Field of the Invention

The field of this invention is phage display of polypeptides. More particularly, this invention relates to a  
20 modified filamentous phage that allows for displaying polypeptides on the surface in a range of densities.

Background of the Invention

Phage display of antibodies was initially based on  
25 systems developed for the display of peptides (Smith, *Science* **228**, 1315-7, 1985). Antibody single chain variable domains were fused to the coat protein gene (gpIII), (McCafferty, et al., *Nature* **348**, 552-554, 1990) resulting in all the gpIII molecules displaying fusion antibodies. However, the fusion of  
30 a polypeptide to the gpIII reduced the ability of the phage to infect bacteria and secondly the multivalent display at the tip of the phage resulted in avidity selection rather than affinity discrimination. Utilizing a phagemid vector (to present the gpIII-fusion) and helper phage rescue (to  
35 introduce the wild type gpIII), the valency of fusion display was reduced and infectivity restored (Bass, et al, *Proteins: Structure, Function, and Genetics* **8**, 309-314, 1990). Likewise, the display of heterodimeric polypeptides such as

antibody F(ab) fragments as either major (gpVIII) (Kang, et al., *Proc. Natl Acad. Sci. USA* **88**, 4363-66, 1991) , or minor (gpIII) (Barbas, et al., *Proc. Natl. Acad. Sci. USA* **88**, 7978-82, 1991; Garrard, et al., *Bio/Technol.* **9**, 1373-77, 1991)

5 coat protein fusions has successfully utilized phagemid with helper phage rescue.

Phage display of antibody fragments and other polypeptides has gained acceptance as a useful tool in

10 contemporary molecular immunology. The density of polypeptide display per filamentous phage particle is influenced by the choice of which phage coat protein is used as fusion partner and the type of vector system used. Molecules expressed from nucleotide sequences fused with the sole copy of gpIII on the

15 phage genome such as fd or M13 resulted in a multivalent cluster display (tri-penta valent) and reduced infectivity of bacteria (McCafferty et al., *Nature* **348**, 552-554, 1990; Smith, *Science* **228**, 1315-7, 1985). Multivalent binding of phage with ligand would favor avidity selection and limit the ability to

20 discriminate between modest gains in affinity (Cwirla, et al., *Proc. Natl Acad. Sci. USA* **87**, 6378-82., 1990). This may be desirable when attempting to isolate ligand binding molecules of lower affinity. Phagemid vectors encoding phage coat protein fusion polypeptides used in conjunction with helper

25 phage rescue, generated phage with restored infectivity and reduced valency permitting enrichment for high affinity interactions (Bass, S., et al. *Proteins: Structure, Function, and Genetics* **8**, 309-314, 1990).

30 Both the high and low density display systems have uses in accessing ligands against target receptors or tissues. It would be desirable to create a phage display system in which the density of the displayed fusion moieties on the phage particle could be modulated from a few displayed copies to

35 less than 1 per phage. To achieve this with existing vectors requires shuttling of inserts between gpIII phage and gpVIII/gpIII phagemid vectors. However this may also be

attained by utilizing a single M13 phage based vector with a synthetic second copy of the gene encoding gpIII or gpVIII (i.e. pseudo wild type) as a fusion partner (Huse, et al., *J. Immunol.* **149**, 3914-20, 1992), and manipulating the phage growth conditions to favor low or moderate rates of fusion incorporation into the phage filament. Incorporating the display expression cassette onto the phage genome may also have the added benefits of fusion expression being synchronous with phage morphogenesis. The present invention describe a phage vector in which a polypeptide is displayed on the phage surface. This display system was used to investigate the modulation of display fusion on phage resulting in optimal phage display as determined by relative panning enrichment efficacy.

#### Brief Summary of the Invention

The present invention discloses a phage vector for the display of polypeptides on the surface of a modified filamentous phage which permits facile manipulation of the valency of display. The gene encoding the polypeptide is fused to a synthetic copy of a major coat protein gene which permits incorporation into the phage during assembly of the filament.

Thus, in one aspect, the present invention provides a modified filamentous phage expression vector. That vector includes a gene encoding a wild type major coat protein of the phage; a leaky, inducible promoter; a gene encoding a synthetic major coat protein of the phage; and a directional cloning site for receiving a nucleotide insert. The insert is a nucleotide that includes a sequence that encodes a translation initiation site, contains a leader sequence that directs polypeptide expression to a bacterial periplasmic membrane and a polypeptide encoding sequence. The directional cloning site is situated between the promoter and the gene encoding the synthetic major coat protein of the phage, such that the polypeptide is expressed as a fusion protein with the synthetic major coat protein.

In preferred embodiments, the translation initiation site is a ribosome binding site, the promotor is the *lac* promoter, the leader sequence is *ompA*, the wild type major coat protein of the phage is gpVIII, the synthetic major coat protein of the phage is a synthetic gpVIII, and the polypeptide is a ligand-binding heterodimeric antibody. An especially preferred filamentous phage is M13. A preferred modified M13 vector of this invention is designated herein as JC-M13-88.

Preferably, the nucleotide insert of the modified filamentous phage is obtained from a pre-selection open reading frame expression and secretion plasmid (pORFES), preferably pORFES II or pORFES IV.

In a related aspect, the present invention provides a process for expressing a polypeptide. The process includes the steps of (a) inserting a nucleotide sequence containing a translation initiation site encoding region, a leader sequence that targets expression of a polypeptide to a bacterial periplasmic membrane and a polypeptide coding sequence that into a directional cloning site of a filamentous phage that contains a gene encoding a wild type phage major coat protein, an inducible promoter and a gene that encodes a synthetic phage major coat protein wherein the directional cloning site is located between the inducible promoter and the gene encoding the synthetic phage major coat protein; and (b) propagating the filamentous phage from step (a) in a bacterium. Preferred translation initiation sites, promoters, leader sequences, polypeptides and major coat proteins are the same as set forth above. A preferred directional cloning site comprises a pair of restriction enzyme sites. Exemplary such enzyme sites are *XbaI* and *HindIII*.

The density of the polypeptides displayed on phage may be modulated by phage altering growth conditions. Propagation is preferably carried out at a temperature of from about 25°C to about 37°C in the absence or presence of inducers that induce

expression by way of the inducible promoter. Lowering the temperature of phage propagation reduced the overall phage yield, yet increased the quality of the antibody display. Likewise the addition of inducers during phage propagation reduced the phage yield but led to enhanced the recovery of phage during panning.

#### Brief Description of the Drawings

In the drawings that form a portion of the specification.

FIG. 1 shows a schematic illustration of JC-M13-88 phage display system. The  $V_H/K$  is inserted into the JC-M13-88 vector from pORFES as an *XbaI-HindIII* fragment. The  $V_H/K$  molecule is expressed as a fusion with the product of a synthetic gpVIII gene, which is not homologous with the *wild-type* gene present in the phage genome. Expression from dual ribosome binding sites (RBS) is controlled by the *Lac* promoter and the  $V_H/K$ -gpVIII fusion molecule is targeted for secretion through the bacterial membrane by the *ompA* leader sequence. The single stranded phage DNA is encapsulated by the *wild type* gpVIII and one or more  $V_H/K$ -gpVIII fusion molecules may also be incorporated into the phage protein coat.

FIG. 2 shows a schematic drawing of a pre-selection open reading frame expression and secretion plasmid (pORFESIV) and a partial nucleotide sequence of that plasmid.

FIG. 3 shows an analysis of  $VH/k$ -gpVIII incorporation into phage filament using electron microscopy. DB3 R100 antibody phage were grown at 25C in the presence of IPTG. Phage were applied to grids and probed with PHS-BSA gold conjugates. The distribution of gold particles per phage was determined by visual examination of phage particles.

FIG. 4 shows the nucleotide sequence (SEQ ID NO:1) of a modified filamentous phage of this invention.



FIG. 5 shows the nucleotide sequence (SEQ ID NO:2) of pORFES II.

5 FIG. 6 shows the nucleotide sequence (SEQ ID NO:3) of pORFES IV.

### Detailed Description of the Invention

#### I. The Invention

10 This invention provides a modified filamentous phage and methods for using that phage to express and display polypeptides on its surface at varying density.

#### II. Modified Filamentous Phage

15 As is well known in the art, a filamentous bacteriophage, hereinafter a filamentous phage, is a member of a group of related viruses that infect bacteria. The term filamentous refers to the long, thin particles of an elongated capsule that envelope deoxyribonucleic acid (DNA) (the phage genome). Well known filamentous phage include fd, f1 and M13. Mature capsules of filamentous phage contain five encoded gene products known as coat proteins: gpVIII, gpIII, gpIV, gpVII and gpIX.

25 A modified filamentous phage of the present invention comprises a gene encoding a wild type phage coat protein and a gene encoding a synthetic form of a coat protein. In a preferred embodiment, the phage contains a gene that encodes a wild type coat protein and a gene that encodes a synthetic form of that same coat protein. In an especially preferred embodiment, the coat protein is major coat protein gpVIII.

35 The modified phage also contains an inducible promoter. Inducible promoters are well known in the art. An especially preferred such well known inducible promoter is the *lac* promoter. Situated between the promoter and the gene for the synthetic coat protein is a directional cloning site designed for receiving a nucleotide insert. As used herein the phrase

"directional cloning site" indicates an insertion site that orients the insert such that expression of a polypeptide coding sequence in the insert will be under the control of the promoter and the polypeptide will be expressed as a fusion protein with the synthetic coat protein. A preferred directional cloning site is a pair of restriction enzyme sites that are the same as restriction enzyme sites at the ends of the nucleotide insert. An exemplary and preferred such restriction enzyme site pair is *XbaI* and *HindIII*.

The nucleotide insert to be received by the modified phage comprises a translation initiation site, a leader sequence and a polypeptide coding region. Translation initiation sites are well known in the art. An exemplary and preferred such initiation site is a ribosome binding site. Ribosome binding sites for use in filamentous phage are well known in the art (See, e.g., U.S. Patent No. 5,658,727, the disclosure of which is incorporated herein by reference). A leader sequence is located upstream to the polypeptide coding region and acts as a signal that targets the polypeptide to the periplasmic membrane of a bacterium. Such leader, signal sequences and their use with filamentous phage are well known in the art (See, e.g., U.S. Patent No. 5,658,727, the disclosure of which is incorporated herein by reference). An exemplary and preferred leader sequence is *ompA*.

In a preferred embodiment, the polypeptide is a ligand-binding heterodimeric receptor. Preferred heterodimeric receptors include immunoglobulins, major histocompatibility antigens of class I or II, lymphocyte receptors, integrins and the like heterodimeric receptors. Immunoglobulins (antibody molecules) can be in the form of Fab or Fv fragments, or other portions of an antibody molecule that contain regions of the variable domain of the heavy and light chains.

Preferably the receptor produced by the subject invention is heterodimeric and is therefore normally comprised

of two different polypeptide chains, which together assume a conformation having a binding affinity, or association constant for the preselected ligand that is different, preferably higher, than the affinity or association constant of either of the polypeptides alone, i.e., as monomers. The heterodimeric receptor is referred to as a ligand-binding heterodimeric receptor to connote its ability to bind ligand.

One or both of the different polypeptide chains is preferably derived from the variable region of the light and heavy chains of immunoglobulin. Typically, polypeptides comprising the light ( $V_L$ ) and heavy ( $V_H$ ) variable regions are employed together for binding a preselected ligand.

A nucleotide insert for use with a modified phage of this invention can be prepared using any means known in the art. In a preferred embodiment, the insert is obtained from an open reading frame expression secretion vector designated herein as pORFES. A schematic illustration of one pORFES (pORFES IV) is shown in FIG. 2. Nucleotide sequences of preferred pORFES are shown in FIG. 5 (pORFES II, SEQ ID NO:2) and FIG. 6 (pORFES IV, SEQ ID NO:3).

As shown in FIG. 2, pORFES IV contains the  $\beta$ -lactamase gene preceded by the ompA leader sequence (underlined) with a 4xgly 1xser linker (underlined). An additional base between the leader and the linker results in a frame shift of the  $\beta$ -lactamase. A NaeI site between the leader sequence and the linker allows subcloning of blunt ended DNA. The CAT gene in the vector backbone permits the vector propagation using chloramphenicol if the vector does not contain an insert restoring the frame of the  $\beta$ -lactamase. Only the ligation of inserts restoring the frame of the  $\beta$ -lactamase which do not contain a stop codon should result in carbenicillin resistance. The XbaI/HindIII cloning sites permit cloning into compatible sites of a modified phage display vector such as JC-M13-88.

Gel-purified polypeptide coding sequences are digested with 70 ng DNase containing  $MnCl_2$ , and the reaction terminated by adding EDTA. After precipitation, the fragments are treated with mung bean nuclease. The resulting mixture containing blunt ended fragments is ligated into restriction enzyme digested pORFES between the ompA leader sequence and the  $\beta$ -lactamase gene. Only the inserts which restore the correct open reading frame and are readily translocated into the periplasm along with the fused  $\beta$ -lactamase confer  $\beta$ -lactam antibiotic resistance. The ligation products are transfected into non-suppressing *E.coli* via electroporation and propagated overnight in Super Broth containing 100  $\mu g/ml$  carbenicillin at 37°C. The transfection efficiency is monitored by plating aliquots on agar plates containing chloramphenicol with or without 100 carbenicillin. The pORFES library DNA is recovered from the overnight culture and inserts released by digestion with *XbaI* and *HindIII*.

A preferred modified filamentous phage of this invention is designated herein as JC-M13-88. A schematic illustration of this phage is shown in FIG. 1 and the nucleotide sequence (SEQ ID NO:1) of this phage is shown in FIG. 4.

The present invention further provides a kit for the expression of a polypeptide. The kit comprises a modified filamentous phage of this invention together with instructions for use of that phage. The kit can further include solvents and reagents for the use of that phage. A preferred phage for inclusion in the kit is the same as set forth above.

### III. Uses of Modified Filamentous Phage

In a related aspect, the present invention provides a process for expressing a polypeptide. The polypeptide is expressed on the surface of a modified filamentous phage as set forth above. The polypeptide is expressed as a fusion protein with a synthetic form of a major coat protein of the

phage. Suitable such coat proteins are disclosed above.

5 The process includes the step of inserting a nucleotide sequence containing a ribosome binding site encoding region, a leader sequence that targets expression of a polypeptide to a bacterial periplasmic membrane and a polypeptide coding sequence that into a directional cloning site of a filamentous phage that contains a gene encoding a wild type phage major coat protein, an inducible promoter and a gene that encodes a synthetic phage major coat protein. The directional cloning site is located between the inducible promoter and the gene encoding the synthetic phage major coat protein. The process further includes the step of propagating the filamentous phage in a bacterium. Preferred promoters, leader sequences, 15 polypeptides and major coat proteins are the same as set forth above. A preferred directional cloning site comprises a pair of restriction enzyme sites. Exemplary such enzyme sites are *XbaI* and *HindIII*. A detailed description of expressing an antibody polypeptide in accordance with the present process 20 can be found hereinafter in the Examples.

The density of the polypeptides displayed on phage using a process of this invention can be modulated by altering growth conditions. Propagation is preferably carried out at a 25 temperature of from about 25°C to about 37°C in the absence or presence of inducers that induce expression by way of the inducible promoter. Lowering the temperature of phage propagation reduced the overall phage yield, yet increased the quality of the antibody display. Likewise the addition of 30 inducers during phage propagation reduced the phage yield but led to enhanced the recovery of phage during panning. The present process does not require the use of a helper/plasmid and has advantages over existing methods of phage display.

35 Using a present process, the integration of  $V_H/K$ -gpVIII fusion(s) has no apparent influence on phage infectivity, and labeling studies suggested that the recombinant molecules are

incorporated at a low density. Other groups have estimated the level of incorporation into the phage filament of polypeptides expressed as fusion with either gpIII or gpVIII using indirect methods (Bass, S., et al. *Proteins: Structure, Function, and Genetics* **8**, 309-314, 1990; Garrard, et al., *Bio/Technol.* **9**, 1373-77, 1991; Zhong, et al., *J. Biol. Chem.* **269**, 24183-8, 1994). The values obtained represented the average number of coat protein fusion's within a phage population. Using electron microscopy with immunogold labeling the distribution of coat protein fusion's on phage prepared under conditions of higher density display was estimated. The maximum number of labels per JC-M13-88 phage particle was four, which is in contrast to earlier studies on phagemid pComb8 /helper in which as many as 24 F(ab)-gpVIII molecules were displayed on the phage particles (Kang, A. S., et al., *Proc. Natl Acad. Sci. USA* **88**, 4363-66, 1991). Using a phagemid/helper system the interval between fusion expression and helper rescue may be extended in early stages of phage preparation, thus permitting accumulation of fusion prior to phage production which permits some phage particles to take up as many as 20 copies of the fusion moiety (both helper and phagemid). In the phage only system the fusion moiety and phage morphogenesis are in concert which would preclude the incorporation of fusion approaching the extent that can be achieved with phagemids prior to helper rescue, additionally only phage displaying the fusion would encode it. One feature observed with JC-M13-88 display vector was the retention of intact inserts throughout the duration of the experiment in contrast to fusion's with gpVIII or III in phagemid vectors. In cells infected with JC-M13-88 phage the metabolic demand on infected cells is high, the addition of fusion-gpVIII molecules driven from a much weaker *Lac* promoter may not significantly perturb this demand, thus loss of insert expression may not confer any discernible growth advantage. However, in general it has been acknowledged that large foreign DNA inserts are often unstable in M13 based vectors (see discussion in Sambrook, et al., *Molecular Cloning: A*

laboratory manual, 1989). Reducing the size of the foreign insert and of homologous nucleotide sequences within the phage genome may confer insert/phage stability. This was achieved in JC-M13-88 by replacing the *Lac Z* polypeptide of M13 mp18 with a non-phage leader sequence (McGuinness, et al., *Nature Biotech.* **14**, 1149-54, 1996) and a synthetic second copy of the gpVIII.

Parameters influencing the yield of phage have been linked closely to conditions favoring bacterial growth. Optimal growth conditions for the bacteria should provide optimal production conditions for phage. Growth conditions during phage preparation such as temperature of phage production and induction of fusion antibody expression by IPTG were investigated. Lowering the temperature of phage propagation reduced the overall phage yield, yet increased the quality of the antibody display. Likewise the addition of IPTG during phage propagation reduced the phage yield but led to enhanced the recovery of phage during panning. The combination of lower temperature and the presence IPTG during phage propagation improved the recovery of phage in panning experiments by more than 100 fold relative to phage prepared at 37° C in the absence of IPTG. The reduction in growth temperature and addition of IPTG may have a concerted effect on phage production and on antibody fusion assembly resulting in a higher level of display per phage particle. Enhancing the density of display in early rounds of panning may be beneficial in accessing lower affinity interactions. By adjusting the growth conditions in later panning rounds the qualitative features of the phage population may be altered to favor "lower" density display. A priori requirement for successful phage display of a polypeptide is the successful production, secretion and assembly of recombinant proteins in *E. coli* which can be influenced by a number of factors in addition to amino acid sequence (for reviews see Marston, et al, *Escherichia coli*. *Biochem. J.* **240**, 1-12, 1986; Schein, *Bio/Technol.* **7**, 1141-9, 1989). Reducing the temperature may

facilitate the correct folding and assembly, and contribute to stability, of the displayed antibody. The present data show that producing a well characterized antibody phage at lower temperatures in the presence of IPTG increases the number of phage recovered in panning experiments, and increases the number of V<sub>H</sub>/k-gpVIII polypeptides incorporated into the phage filament. At all growth temperatures investigated, phage produced in the presence of IPTG were more readily recovered in panning experiments than phage produced without IPTG induction. In contrast, experiments with antibody-gpVIII phage produced using a phagemid / helper phage system the opposite situation was found (Kretzschmar & Geiser, *Gene* **155**, 61-65, 1995). Hence by manipulating the propagation temperature and the induction of the fusion moiety it should be possible to prepare phage for accessing based on avidity (higher density display, 25°C, + IPTG) or affinity (lower density display, 37°C, - IPTG).

The present process was also evaluated for the ability to discriminate between two variants of the antibody DB3 (W100 and R100), with widely disparate affinities for PCMO-BSA yet indistinguishable affinities for PHS-BSA. The recovery of DB3 R100 over W100 on either PHS-BSA or CMO-BSA implies that R100 has a higher affinity for both ligands.

The Examples that follow illustrate preferred embodiments of the present invention and are not limiting of the specification or claims in any way.

#### **EXAMPLE 1: Phage Construction**

***Alphabetical list of oligonucleotides.*** (restriction sites underlined),

DB3BLAB 5'-TCT AGA AGC TTG CCC ACC CTC ATT CCT GTT GAA GCT-3'

(SEQ ID NO:4)

JC102 5'- GGC GCT GCT AGC GTA GCT CAG GCT CAG GTG AAA CTG CTC GAG-3' (SEQ ID NO:5)

M13-JC117 5'-TCA TCA TAC TAG TGA TGG CGT TCC TAT TGG T-3' (SEQ



ID NO:6)

M13-JC118 5'-AAG CTT ATG ATG TCT AGA GCT GTT TCC TGT GTG AA-3'  
(SEQ ID NO:7)

SYNVIII-JC119 5'-TAA GCT TCT GGC GCC GTC CCT GCA GAA GGT GA-3'  
(SEQ ID NO:8)

SYNVIII-JC120 5'-AAG CTA GCT TAA AAA AAA GCC CGC-3' (SEQ ID  
NO:9)

**Construction of modified M13.** Nucleotide sequence of  
bacteriophage M13mp18 (Messing, *Methods Enzymol.* **101**, 20-79,  
1983; Messing, *Gene* **100**, 3-12, 1991; Yanisch-Peron, et al.,  
*Gene* **33**, 103-119, 1985) , was amplified (94°C for 2 min, 10  
cycles; 94° C for 30 s, 60°C for 1 min, 68°C for 6 min,  
followed by 20 cycles during which the extension time was  
increased by 20 s per cycle) from replicative form DNA using  
primers M13-JC117, M13-JC118 and the Expand™ Long Template PCR  
System (Boehringer Mannheim). The PCR amplified M13mp18 was  
treated sequentially with T4 DNA polymerase, T4 polynucleotide  
kinase, and finally T4 DNA ligase, using standard methods  
(Sambrook, et al., *Molecular Cloning: A laboratory manual*,  
1989). *E. coli* XL1-Blue strain (Stratagene) were transformed  
with the ligated DNA and a standard M13 plaque assay was  
performed in the presence of IPTG and X-gal as described  
(Sambrook, et al., *Molecular Cloning: A laboratory manual*,  
1989). Replicative form DNA was prepared from colorless  
plaques and the desired DNA construct identified by analyzing  
restriction enzyme digests. DNA coding for a pseudo gpVIII,  
which is not homologous to the wild-type gene but nearly  
identical in primary sequence, was PCR amplified (30 cycles;  
94°C for 30 s, 55°C for 1 min, 72°C for 1 min) from vector  
f88-4 ((Zhong, et al., *J. Biol. Chem.* **269**, 24183-8, 1994)  
provided by G. P. Smith, Univ. Missouri, MO) using primers  
SynVIII-JC119 and SynVIII-JC120. The PCR product was cloned  
using the TA Cloning kit plasmid pCR™II (Invitrogen), and the  
sequence of the DNA insert was confirmed by DNA sequencing.  
DNA coding for the synthetic gpVIII was ligated into the  
modified M13. The modified phage containing the wild type and

the pseudo wild type gpVIII was designated JC-M13 -88.

**Construction of DB3 V<sub>H</sub>/k Phage.** The wild type anti-progesterone antibody DB3 (He, et al., *Immunology* **84**, 662-668, 1995; Wright, et al., *Nature* **295**, 415-417, 1982) has a tryptophan residue at position 100 of the V<sub>H</sub> domain (designated W100). In a mutant single chain V<sub>H</sub>/k molecule position 100 is arginine (designated R100). The DNA sequences coding for the DB3 V<sub>H</sub>/k W100 (He, et al., *Immunology* **84**, 662-668, 1995) and R100 variants were PCR amplified using the oligonucleotide primers JC102 and DB3BlaB and ligated into the plasmid pORFES (open reading frame / expression / secretion as *Nhe* - *HindIII* fragments, and the ligation products were transformed into bacteria. The plasmid pORFES is a low copy number plasmid in which sequences are expressed as fusions with beta lactamase. Antibiotic selection has been used to screen libraries of peptides and antibody genes fused with the *bla* gene for open reading frames and successful periplasmic targeting in bacteria. Here, the sub-cloning of the DB3 via pORFES was necessary both to introduce restriction enzyme sites and to provide a second ribosome binding site and the *ompA* leader sequence for the correct expression of the DNA insert. The 1.1 kilo base pair DNA fragments coding for the DB3 V<sub>H</sub>/k variants were excised from pORFES using *XbaI* and *HindIII*, then ligated into *XbaI* / *HindIII* treated JC-M13-88. Phage encoding and displaying the W100 and the R100 V<sub>H</sub>/k molecules were generated.

**Western blotting.** M13mp18 phage or DB3 R100 phage were precipitated with PEG and re suspended in SDS PAGE sample buffer (Laemmli, *Nature* **277**, 680-685, 1970). Polypeptides from  $1 \times 10^{12}$  pfu were fractionated on 12.5 % polyacrylamide gel, then transferred electrophoretically to nitrocellulose paper (NCP) (Towbin, et al., *Proc. Natl Acad. Sci. USA* **76**, 4350-4354, 1979). The NCP was incubated firstly in blocking buffer (4% bovine serum albumin [BSA] in phosphate buffered saline [PBS],

0.02 %  $\text{NaN}_3$ ), then with goat anti-mouse kappa light chain alkaline phosphatase conjugate (Southern Biotech). After washing NCP in PBS 0.05% Tween-20 (PBS/T) labeled polypeptides were revealed using a chromogenic substrate as described (Harlow & Lane, *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory Press, New York, 1988).

Example 2: Phage Propagation

**Phage panning experiments.** Unless stated otherwise

phage were propagated in *Escherichia coli* (strain XL1-blue, Stratagene) in Superbroth ([3-N-morpholino] propane-sulfonic acid, 10 g  $\text{l}^{-1}$ , Bacto tryptone, 30 g  $\text{l}^{-1}$ , yeast extract, 20 g  $\text{l}^{-1}$ , pH 7.0) at 37°C in for 16 h, and recovered from the spent media using PEG-8000 as described (Sambrook, et al., *Molecular Cloning: A laboratory manual*, 1989). Multiwell removable strips (Maxisorp, Nunc) were coated overnight at room temperature (RT) with 100  $\mu\text{l}$  / well PCMO-BSA, PHS-BSA, or goat anti-mouse kappa light chain IgG (Southern Biotech), each diluted to 10  $\mu\text{g ml}^{-1}$  in 0.1 M carbonate buffer (pH 9.6). The coating solution was replaced with blocking buffer and after 2 h at RT 100  $\mu\text{l}$  of phage diluted in 0.1% BSA in PBS were added to duplicate wells and incubated for 3 h at RT. Wells were washed x5 (5 min per wash) with PBS/T plus 0.1% BSA, and bound phage were eluted into 100  $\mu\text{l}$  / well 0.1 M glycine (pH 2.2) for 15 min and neutralized by adding an equal volume of 1 M Tris-HCl (pH 8.0). In experiments requiring multiple rounds of phage panning against antigen the neutralized eluate was added to freshly prepared bacteria in order to generate phage required for the next round of panning. Phage titer was assessed using standard methodology as described (Sambrook, et al., *Molecular Cloning: A laboratory manual*, 1989).

**Oligonucleotide hybridization and plaque immunostaining procedures.** Replicas of phage plaques were made by applying dry nitrocellulose filters (0.45  $\mu\text{m}$ , Schleicher & Schuell) to agar plates. After 1 min filters were removed, air dried, then baked in a vacuum oven at 80°C for 1 h. Filters were incubated in pre-hybridization buffer (0.25 % non fat milk powder in 6 x

SSC) at 68°C for 1 h. Two 15 bp oligonucleotides  
GAAGTAACGGTTGAC (SEQ ID NO:10) and GAAGTACCAGTTGAC (SEQ ID  
NO:11) , designed to hybridize in a specific fashion to the  
DB3 R100 and W100 variants respectively, were end-labeled with  
5 digoxigenin using the Genius 5 3' end labeling kit (Boehringer  
Mannheim) according to the manufacturers instructions. Labeled  
probes were added to the filters in pre hybridization buffer  
and incubated at 25°C for 4 h. After washing with fresh pre  
hybridization buffer the filters were first incubated in  
10 blocking buffer, then transferred to PBS/T containing  
alkalinephosphatase conjugated anti-digoxigenin antibodies  
(Boehringer Mannheim). In plaque immunostaining experiments,  
nitrocellulose plaque replicas on nitrocellulose were made as  
above except the filters were left on the plates for 2 h at  
15 37°C before removing and blocking as above. Blocked filters  
were incubated with alkaline phosphatase conjugated anti-mouse  
or anti-human k light chain antibodies (Southern Biotech). All  
filters were washed and developed as described for western  
blotting except in hybridization experiments the development  
20 was carried out overnight at 4°C.

**Electron Microscopy** . Stable complexes of 5 nm gold  
particles with PHS-BSA or goat anti mouse kappa chain were  
prepared essentially as described (Horisberger & Rosset, *J.*  
25 *Histochem. Cytochem.* **25**, 295-305, 1977; Slot, & Geuze, *Eur. J.*  
*Cell Biol.* **38**, 87-93, 1985). To ensure the removal of any  
unbound protein the complexes were centrifuged through a 7%  
glycerol cushion as described (Slot, & Geuze, *Eur. J. Cell*  
*Biol.* **38**, 87-93, 1985). The pelleted complexes were re  
30 suspended in PBS containing 0.1% BSA and 0.02% NaN<sub>3</sub>, and stored  
at 4°C. Phage were prepared at 25°C with IPTG or at 37°C  
without IPTG diluted into PBS containing 1% BSA and  $2 \times 10^9$   
pfu were applied to Formvar coated nickel grids (200 mesh).  
Unbound phage were removed by washing with PBS and then 1% BSA  
35 in PBS was applied for 10 min. Protein-gold complexes were  
centrifuged briefly (700 x g , 2 min.), and applied to the  
grids undiluted (ca. 20 µg protein ml<sup>-1</sup>). After 30 min. the

5 grids were washed with PBS, then stained with 1% uranyl acetate for visualization by electron microscopy. All incubations were done at RT in a humid atmosphere. Randomly selected areas on the grids were photographed in order to quantify the number of gold particles associated with the phage.

10 The JC-M13-88 display vector was constructed by modifying M13mp18. The b-galactosidase  $\alpha$ -complementation peptide encoding region was replaced with the ompA leader, antibody V<sub>H</sub>/k and the synthetic gpVIII cassette as shown in Figure 1.

15 Western blots of poly ethylene glycol (PEG)-precipitated antibody phage polypeptides probed with anti-mouse k light chain reagent revealed a single immunoreactive protein which migrated at ~ 47 kilodaltons (kDa), which is similar to the predicted molecular weight of the DB3 R100 V<sub>H</sub>/k-gpVIII polypeptide (44,656 Daltons).

20 Polypeptides from M13mp18 control phage were not immunoreactive. This finding confirmed that the DB3 V<sub>H</sub>/k-gpVIII moiety was successfully integrated into the phage particle (or at least coprecipitated with phage). Phage prepared at 25°C with IPTG induction were used to determine the density of the

25 antibody on the filament surface by electron microscopy. The number of progesterone-11 $\alpha$ -ol-hemisuccinate-bovine serum albumin (PHS-BSA) labels per phage varied from one (~34% phage) to four (~1% phage) adopting a classical distribution pattern. About 50% of the phage did not display functional

30 antibody. Whilst phage prepared at 37°C without IPTG, were essentially devoid of fusion and the few phage that could be observed binding antigen or binding to goat anti-mouse kappa chain were monovalent (3b&c). Preparations of wild-type M13mp18 phage were not labeled by either the PHS-BSA or the

35 BSA gold probes. The effect of growth temperature and fusion induction by IPTG on antibody phage yield and subsequent enrichment by binding to immobilized steroid was investigated. Lower growth temperature resulted in lower

overall phage production. Cultures grown for 6 h at 37 °C or 16 h at 30 °C had similar titers of phage ( $\sim 10^{12}$  plaque forming units (pfu)  $\text{ml}^{-1}$ ), while cultures grown for 16 h at 25 °C resulted in a further ten fold reduction in titer. The addition of 1mM IPTG to bacterial cultures reduced the final phage titer after equivalent growth periods by 3 to 4 fold. In a panning experiment with equivalent pfu of phage prepared at 25 °C with IPTG and at 37 °C without IPTG, a 100 fold difference was noted in the phage eluted (Table I).

**Table I.**

Growth conditions for DB3 R100 phage	Pfu Eluted <sup>x*</sup> ( x 10 <sup>4</sup> )	% pfu eluted/ pfu applied	Recovery factor <sup>@</sup>
37 °C +IPTG	5.58 (+/-0.3)	0.0279	5.5
37 °C	1.02 (+/-0.04)	0.0051	1.0
30 °C +IPTG	19.44 (+/-0.8)	0.0972	19.1
30 °C	5.18 (+/-0.2)	0.0259	5.1
25 °C +IPTG	113.96 (+/-11.2)	0.5698	111.7
25 °C	39.22 (+/-2.84)	0.1961	38.5
M13mp18*	0.1 (+/-0.04)	0.0005	0.1

\*DB3R100 or M13mp18 phage were propagated using different growth conditions and  $2 \times 10^8$  plaque forming units were applied to ELISA plates coated with either PCMO-BSA or BSA alone.

After a fixed time interval the plates were washed and bound phage were eluted with acid and the output titer was determined.

\*M13mp18 were propagated at 37 °C without IPTG.

#Figures represent the mean average of plaque forming units eluted from two separate experiments corrected for non-specific binding to BSA (2860 +/- 1690). Values for the experimental error are shown in ().

@The quantity of phage eluted is shown relative to the DB3 R100 phage produced at 37 °C without IPTG, here assigned a

value of 1.

Phage prepared at the lower temperature with IPTG appeared to enrich more efficiently in the panning process. The amount of antibody incorporated into the phage was analyzed by western blotting, The addition of IPTG slightly increased the amount of fusion polypeptide. In phage propagated at 37°C we observed a small amount of an immunoreactive species of approximately 20 kDa. This is likely due to  $V_H/k$ -gpVIII polypeptide proteolysis during phage storage in which the  $V_H/V_L$  has been cleaved. In phage prepared at 30°C and 25°C this band was was not detected. Phage prepared at the lower temperature also appeared to have increased antibody fusion incorporation.

The recombinant wild type steroid binding antibody fragment derived from DB3 encodes a tryptophan at position 100 of the  $V_H$  domain and is designated W100. The two phage W100 and R100 differ in two nucleotides which change the codon at position 100 of the  $V_H$  domain of the displayed DB3 from tgg to cgt (arginine) respectively. The affinities of the recombinant DB3  $V_H/k$  W100 and R100 variants for progesterone and related steroids were determined previously (W100 to PHS-BSA,  $K_a \sim 10^9 M^{-1}$ , and to progesterone-3-carboxymethyloxime (PCMO)-BSA  $K_a \sim 10^7 M^{-1}$ ; the R100 mutant to PHS-BSA,  $K_a \sim 10^9 M^{-1}$ , and to PCMO-BSA  $K_a \sim 10^{11} M^{-1}$ ). However, the authors were not able to distinguish either DB3 variant by binding to PHS-BSA by competition enzyme-linked immunoadsorbent assay (ELISA). A mixture of the W100 and the R100  $V_H/k$  phage was diluted into a 50,000-fold excess of a control phage (BR4) displaying a human  $V_H/k$ , and repeatedly panned on either progesterone PCMO-BSA, PHS-BSA or an anti-mouse kappa light chain antibody. A mock panning of the phage mixture was carried out with plates coated with unmodified BSA. The recovery of phage displaying murine (DB3) or human (BR4)  $V_H/k$  polypeptides was monitored by immunostaining of plaque replica filter lifts on nitrocellulose filters (Table II).

Table II.

Enrichment factor <sup>#</sup> aft r panning on antigen				
Rounds of panning*	PHS-BSA	PCMO-BSA	Anti-mouse kappa Light chain IgG	
0	1	1	1	
1	580	730	640	
2	$8.1 \times 10^4$	$1.8 \times 10^5$	$4.3 \times 10^4$	
3	$5.8 \times 10^5$	$2.6 \times 10^6$	$5.9 \times 10^5$	

\*An equal mixture of the murine DB3W100 and R100 phage was diluted into a 50,000 fold excess of the control phage BR4, which displays a human  $V_H/k$  antibody.  $\sim 1 \times 10^{10}$  mixed phage were used in panning experiments.

#This figure represents the increase in the ratio of DB3 phage over BR4 phage in the eluted phage relative to the initial phage mixture (DB3:BR4=1:50,000), here assigned a value of 1. Phage were propagated at 37°C without IPTG.

The proportion of DB3 phage within the phage mixture was enhanced by over 700 fold following a single round of panning on steroid coated plates. Further rounds of panning increased this proportion although the first round produced the most dramatic increase. This may be due to the saturation of available binding sites during latter rounds of panning by excess DB3 phage. We utilized two independent panning ligands to differentiate between selection based on steroid binding (i.e. a functional combining site) and on anti-kappa light chain binding (i.e. epitope recognition). However, the similar enrichment rate of DB3 phage over the control phage by panning on PHS and PCMO was paralleled by panning against anti-mouse k light chain antibodies. This provides a correlate that the majority if not all of the  $V_H/k$  molecules on the phage which



retain the kappa epitope also retain steroid binding.

The proportion of phage encoding the DB3 R100 or W100 variants was determined by oligonucleotide hybridization to replica plaque filter lifts (Table III).

**Table III**

% DB3 R100 variant in phage eluted after panning on antigen <sup>#</sup>				
Rounds of panning*	PHS-BSA	PCMO-BSA	Anti-mouse kappa Light chain IgG	
0	50+	50	50	
1	nd	nd	nd	
2	99.65	96.95	82.1	
3	100	98.75	79.15	

\*A mixture containing equal numbers of the murine DB3W100 and R100 phage was diluted into a 50,000 fold excess of the control phage BR4, which displays a human V<sub>H</sub>/κ antibody.

~1x10<sup>10</sup> mixed phage were used in panning experiments.

nd - Not determined - After one round of panning the ratio of the murine DB3:human BR4 was too small to permit accurate analysis of this value.

+The initial phage mixture used in this experiment contained an equivalent number of R100 and W100DB3 phage.

#Figures represent the percentage of the murine DB3 phage

eluted with the R100 mutation. Phage were propagated at 37 °C without IPTG.

In order to determine whether the enrichment was a function of steroid-ligand binding and/or a growth advantage of one DB3 variant over the other during propagation, a 1:1

mixture of the two phage in an excess of BR4 (human antibody phage) was panned against anti-mouse  $\kappa$  light chain antibodies. This enrichment being independent of steroid binding should permit other factors influencing phage display to be assessed.

5 After two rounds of panning 82 % of the DB3 phage were the R100 variant, this differential was maintained in subsequent round of panning. Although somewhat removed from the 50 % figure expected, and probably due to a slight bias introduced in the early rounds of panning since the proportion difference

10 did not increase in subsequent rounds. Whilst the proportion of R100 in the eluted phage was significantly greater following panning on both PHS and PCMO steroids and increased with each round of selection. Based upon our knowledge of the binding affinities of the DB3  $V_H/\kappa$  variants for the different

15 steroids, our observation that the R100 variant was preferentially enriched over W100 by panning on PCMO-BSA was not unexpected. However, a similar enrichment after panning on PHS-BSA implies that the DB3 R100 appears to have a slightly higher affinity for this ligand than the DB3 W100. The "mock"

20 panning on BSA did not enrich for DB3 antibody phage over the control phage BR4, confirming that any potential growth advantage over the BR4 phage by either of the DB3 phage variants was not significant. Also, and despite exhaustive analysis of the recombinant phage by immunostaining of plaque

25 replicas filter lifts made throughout the panning experiments, we did not observe loss of the  $V_H/\kappa$  gene. This finding suggests that single chain antibody likely inserts in JC-M13-88 appear to be stable.

30

## WHAT IS CLAIMED IS:

1. A modified filamentous phage expression vector comprising a gene encoding a wild type major coat protein of the phage; a leaky, inducible promoter; a gene encoding a synthetic major coat protein of the phage; and a directional cloning site for receiving an insert that comprises a ribosome binding site encoding region, a leader sequence that directs polypeptide expression to a bacterial periplasmic membrane and a polypeptide encoding region, wherein the directional cloning site is situated between the promoter and the gene encoding the synthetic major coat protein of the phage.
2. The vector of claim 1 wherein the promoter is the *lac* promoter.
3. The vector of claim 1 wherein the wild type major coat protein of the phage is gpVIII.
4. The vector of claim 1 wherein the synthetic major coat protein of the phage is gpVIII.
5. The vector of claim 1 wherein the leader sequence is *ompA*.
6. The vector of claim 1 wherein the filamentous phage is M13.
7. The vector of claim 1 wherein the polypeptide is a ligand-binding heterodimeric antibody.
8. The vector of claim 1 that contains the insert.
9. The vector of claim 1 designated JC-M13-88.

10. The vector of claim 1 having the sequence of SEQ ID NO:1.
- 5 11. The vector of claim 1 wherein the insert is obtained from a pre-selection open reading frame expression and secretion plasmid.
- 10 12. The vector of claim 10 wherein the pre-selection open reading frame expression and secretion plasmid is designated pORFES II or pORFES IV.
13. A process for expressing a polypeptide, the process comprising the steps of:
- 15 a) inserting a nucleotide sequence that contains a ribosome binding site encoding region, a leader sequence that targets expression of a polypeptide to a bacterial periplasmic membrane and a polypeptide encoding region
- 20 into a filamentous phage that contains a gene encoding a wild type phage major coat protein, an inducible promoter a gene that encodes a synthetic phage major coat protein;and
- 25 b) expressing the polypeptide by propagating the filamentous phage from step (a) in a bacterium.
14. The process of claim 13 wherein the leader sequence is ompA.
- 30 15. The process of claim 13 wherein the polypeptide coding sequence encodes a ligand-binding heterodimeric antibody.
- 35 16. The process of claim 13 wherein the inducible promoter is the lac promoter.

17. The process of claim 13 wherein the wild type phage major coat protein is gpVIII.
- 5 18. The process of claim 13 wherein the synthetic phage major coat protein is gpVIII.
19. The process of claim 13 wherein the filamentous phage is M13.
- 10 20. The process of claim 13 wherein the nucleotide sequence insert is obtained from a pre-selection open reading frame expression and secretion plasmid.
- 15 21. The process of claim 20 wherein the pre-selection open reading frame expression and secretion plasmid is designated pORFES II or pORFES IV.
- 20 22. The process of claim 13 wherein the phage resulting from step (a) is propagated at a temperature of from about 25°C to about 37°C.
- 25 23. The process of claim 22 wherein the phage resulting from step (a) is propagated in the absence or presence of an agent that induces the leaky, inducible promoter.
24. The process of claim 23 wherein the leaky, inducible promoter is the *lac* promoter and the agent is isopropyl thio- $\beta$ -D-galactopyranoside.

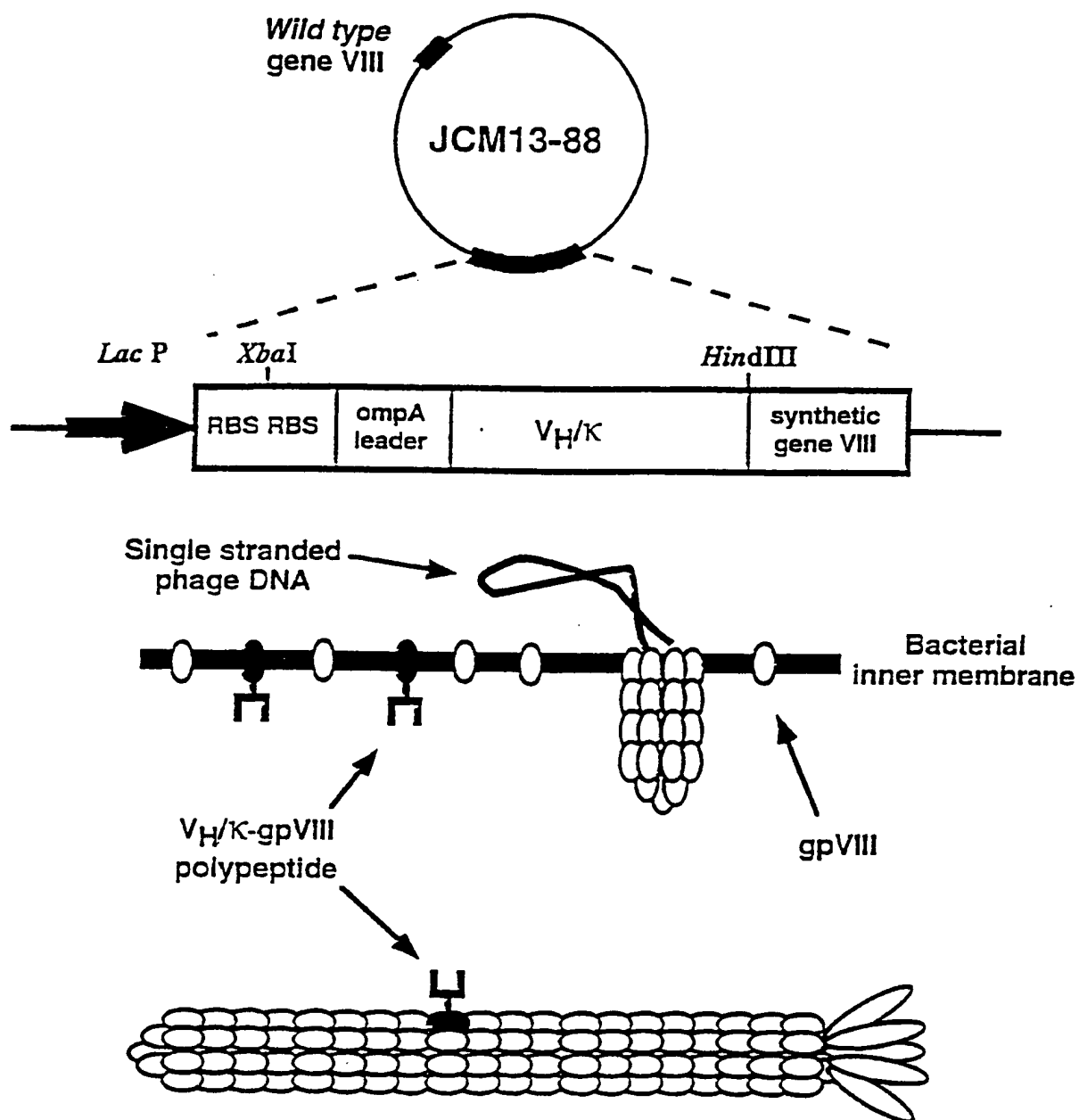


FIG. 1

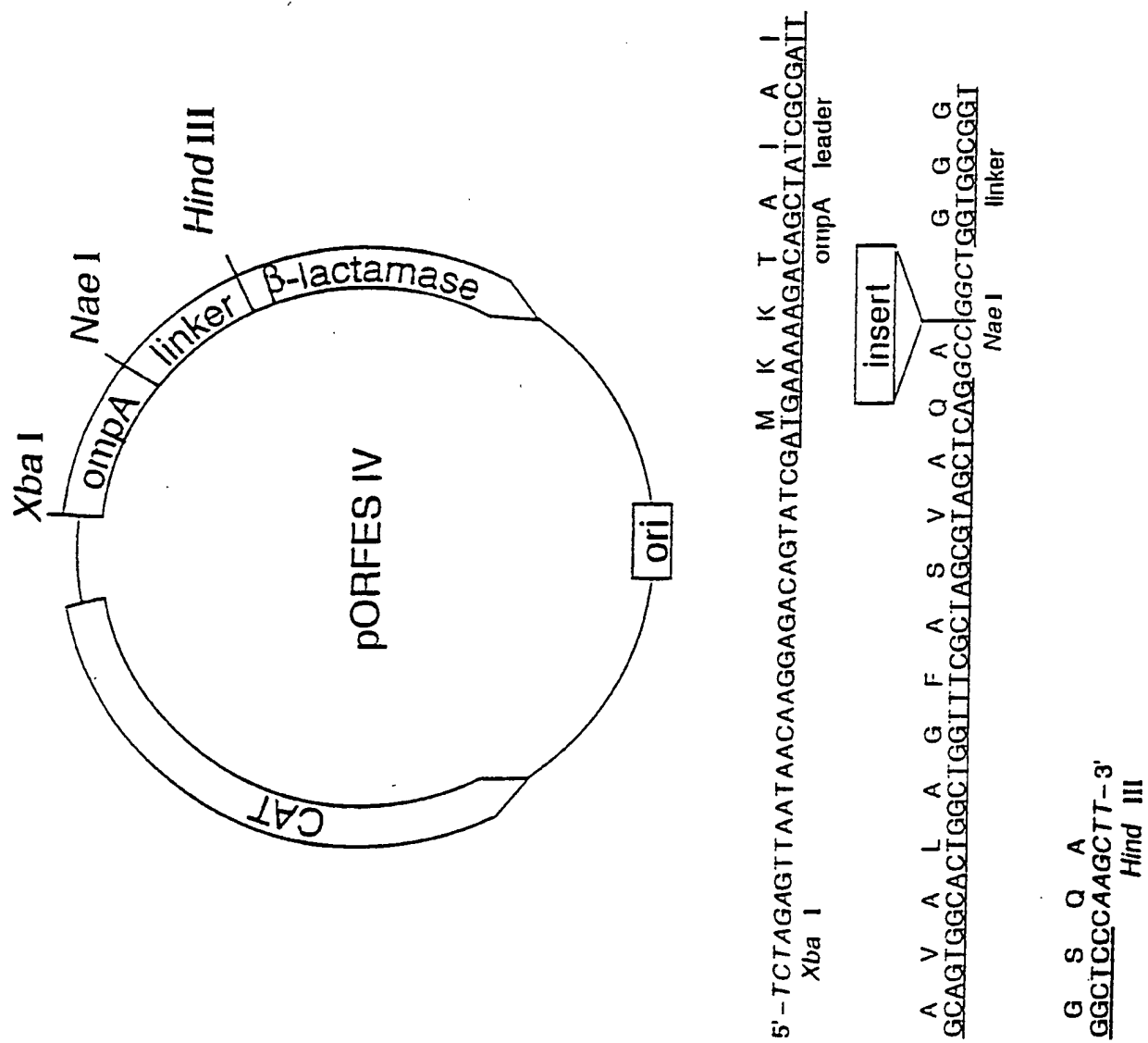


FIG. 2

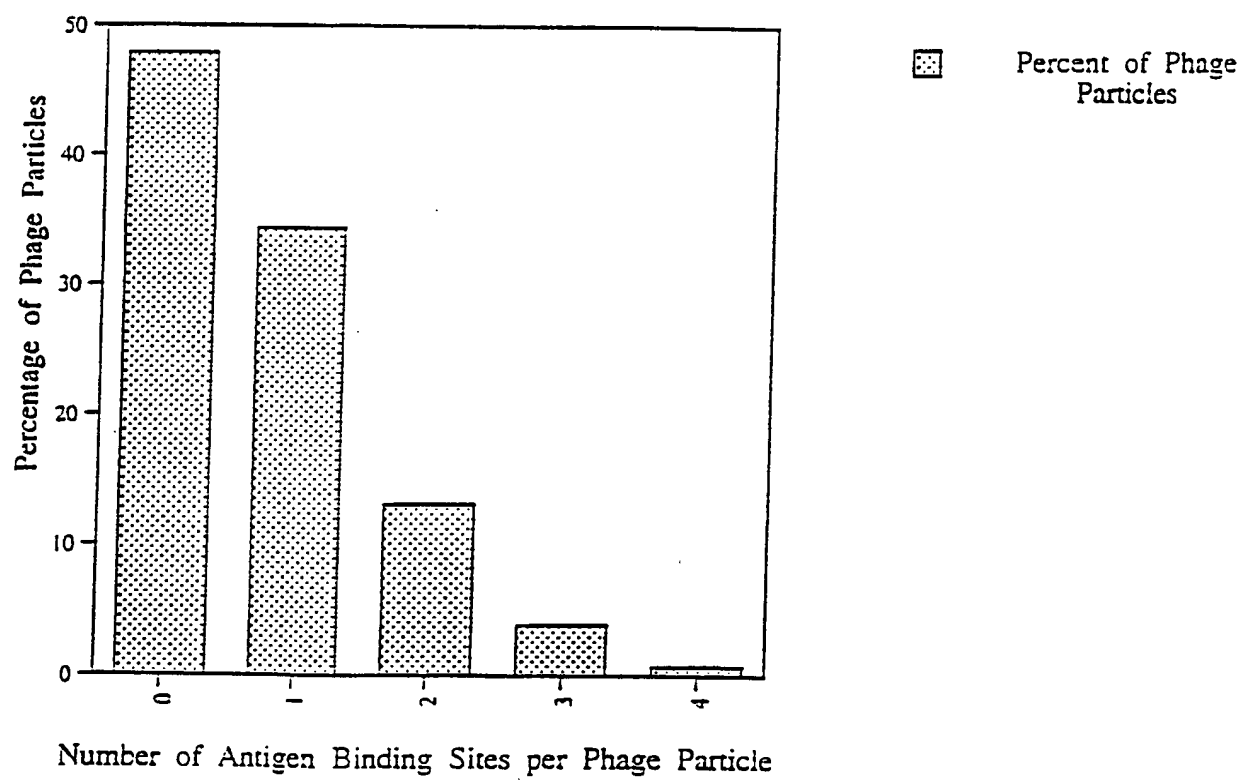


FIG. 3



tctagaGTTAATAACAAGGAGACAGTATCGATGAAAAAGACAGCTATCGCGATTG  
CAGTGGCACTGGCTGGTTTTcgetagcgtagctcagGCCGGCcAAGCTTCTGGCGCCGTC  
CTGCAGAAGGTGATGACCCGGCTAAAGCTGCTTTTGA CTCTTTCAGGCTTCTG  
CTACTGAATACATCGGCTACGCTTGGGCTATGGTGGTTGTTATCGTTGGTGCTA  
CTATTGGCATCAAAC TTTTCAAAAAATTCACTTCTAAAGCGTCTTAATGAACTC  
AGATACCCAGCCCGCCTAATGAGCGGGCTTTTTTTTAAGCTAGTGATGGCGTTC  
CTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACA  
AAATATTAACGTTTACAATTTAAATATTTGCTTATACAATCTTCCTGTTTTTGGG  
GCTTTTCTGATTATCAACCGGGGTACATATGATTGACATGCTAGTTTTACGATT  
ACCGTTTCATCGATTCTCTTGTTTGCTCCAGACTCTCAGGCAATGACCTGATAGC  
CTTTGTAGATCTCTCAAAAATAGCTACCCTCTCCGGCATTAAATTTATCAGCTAG  
AACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGGCCTTTCTCACCC  
TTTTGAATCTTTACCTACACATTACTCAGGCATTGCATTTAAAAATATATGAGGG  
TTCTAAAAATTTTTATCCTTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATT  
ACAGGGTCATAATGTTTTTGGTACAACCGATTAGCTTTATGCTCTGAGGCTTT  
ATTGCTTAATTTTGCTAATTCTTTGCCTTGCTGTATGATTTATTGGATGTTAAT  
GCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAA  
AATATAGCTAAACAGGTTATTGACCATTGCGAAATGTATCTAATGGTCAAAC  
AAATCTACTCGTTCGCAGAATTGGGAATCAACTGTTACATGGAATGAACTTC  
CAGACAC  
CGTACTTTAGTTGCATATTTAAAACATGTTGAGCTACAGCACCAGATTCAGCAA  
TTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAA  
GGTACTCTCTAATCCTGACCTGTTGGAGTTTGCTTCCGGTCTGGTTCCGCTTTGAA  
GCTCGAATTAAAACGCGATATTTGAAGTCTTTCCGGGCTTCCTCTTAATCTTTTTG  
ATGCAATCCGCTTTGCTTCTGACTATAATAGTCAGGGTAAAGACCTGATTTTTG  
ATTTATGGTCATTCTCGTTTTCTGAAC TGTTTAAAGCATTTGAGGGGGATTCAA  
TGAATATTTATGACGATTCCGCAGTATTGGACGCTATCCAGTCTAAACATTTTA  
CTATTACCCCTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTT  
TTATCGTCGTCTGGTAAACGAGGGTTATGATAGTGTTGCTCTTACTATGCCTCG  
TAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTGGTATTCCTAAATCT  
CAACTGATGAATCTTTCTACCTGTAATAATGTTGTTCCGTTAGTTTCGTTTTATTA  
ACGTAGATTTTTCTTCCCAACGTCTGACTGGTATAATGAGCCAGTTCTTAAAA  
TCGCATAAGGTAATTCACAATGATTAAAGTTGAAATTAAACCATCTCAAGCCC  
AATTTACTACTCGTTCTGGTGTCTCTCGTCAGGGCAAGCCTTATTCACTGAATG  
AGCAGCTTTGTTACGTTGATTTGGGTAATGAATATCCGTTCTTGTCAAGATTA  
CTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTGT  
CCTCTTTCAAAGTTGGTCAGTTCCGTTCCCTTATGATTGACCGTCTGCGCCTCGT  
TCCGGCTAAGTAACATGGAGCAGGTGCGGATTTGACACAATTTATCAGGCG  
ATGATACAAATCTCCGTTGTACTTTGTTTCGCGCTTGGTATAATCGCTGGGGGT  
CAAAGATGAGTGTTTTAGTGTAATCTTTTCGCTCTTTTCGTTTTAGGTTGGTGCT  
TCGTAGTGGCATTACGTATTTTACCCGTTAATGGAACTTCCTCATGAAAAAG  
TCTTTAGTCCTCAAAGCCTCTGTAGCCGTTGCTACCCTCGTTCCGATGCTGTCTT  
TCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCC

TCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGC  
GCAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATA  
AACCGAT  
ACAATTAAAGGCTCCTTTTTGGAGCCTTTTTTTTTGGAGATTTTCAACGTGAAAA  
AATTATTATTCGCAATTCCTTTAGTTGTTCCCTTTCTATTCTCACTCCGCTGAAAC  
TGTTGAAAGTTGTTTAGCAAAACCCCATACAGAAAATTCATTTACTAACGTCTG  
GAAAGACGACAAAACCTTTAGATCGTTACGCTAACTATGAGGGTTGTCTGTGGA  
ATGCTACAGGCGTTGTAGTTTGTACTGGTGACGAAACTCAGTGTTACGGTACAT  
GGGTTTCTATTGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTG  
GCGGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTAATAACCTCCTGAGTAC  
GGTGATACACCTATTCCGGGCTATACTTATATCAACCCTCTCGACGGCACTTAT  
CCGCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCT  
CAGCCTCTTAATACTTTTCATGTTTCAGAATAATAGGTTCCGAAATAGGCAGGGG  
GCATTAACCTGTTTATACGGGCACTGTTACTCAAGGCACTGACCCCGTTAAACT  
TATTACCAGTACACTCCTGTATCATCAAAAGCCATGTATGACGCTTACTGGAAC  
GGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAATGAAGATCCATTGCTT  
TGTGAATATCAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTCAATGCTGGC  
GGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGTGGCTCTGAGGG  
TGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTG  
GTTCCGGTGATTTTGATTATGAAAAGATGGCAACGCTAATAAGGGGGCTATG  
ACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCCAACTTGA  
TTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGTTTCC  
GGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGCTGGCTCTAATCCCAA  
ATGGCTCAAGTCGGTGACGGTGATAAATCACCTTTAATGAATAATTCGGTCAA  
TATTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGCTTTAGCGCTG  
GTAAACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTG  
TCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCTACGTTTGCT  
AACATACTGCGTAATAAGGAGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGT  
TATTATTGCGTTTCTCCTCGGTTTCTTCTGGTAACCTTTGTTCCGGCTATCTGCTTAC  
TTTTCTTAAAAAGGGCTTCGGTAAGATAGCTATTGCTATTTCAATTGTTTCTTGCT  
CTTATTATTGGGCTTAACTCAATTCTTGTTGGGTTATCTCTCTGATAATTAGCGCTC  
AATTACCCTCTGACTTTGTTTACGGGTGTTTACGTTAATTCTCCCGTCTAATGCGCT  
TCCCTGTTTTTATGTTATTCTCTCTGTAAAGGCTGCTATTTTCAATTTTGACGTTA  
AACAAAAAATCGTTTCTTATTTGGATTGGGATAAATAATATGGCTGTTTATTTT  
GTAACCTGGCAAATTAGGCTCTGGAAAGACGCTCGTTAGCGTTGGTAAGATTCA  
GGATAAAATTGTAGCTGGGTGCAAAATAGCAACTAATCTTGATTAAAGGCTTC  
AAAACCTCCCGCAAGTCCGGGAGGTTGCTAAAACGCCTCGCGTTCTTAGAATA  
CCGGATAAGCCTTCTATATCTGATTTGCTTGCTATTGGGCGCGGTAATGATTCC  
TACGATGAAAATAAAAACGGCTTGCTTGTTCTCGATGAGTGCGGTACTTGTTTT  
AATACCCGTTCTTGGAATGATAAGGAAAGACAGCCGATTATTGATTGGTTTCTA  
CATGCTCGTAAATTAGGATGGGATATTATTTTCTTGTTTACGGACTTATCTATTG  
TTGATAAACAGGCGCGTTCTGCATTAGCTGAACATGTTGTTTATTGTCGTCGTC  
TGGACAGAATTACTTTACCTTTTGTCCGTACTTTATATTCTCTTATTACTGGCTC  
GAAAATGCCTCTGCCTAAATTACATGTTGGCGTTGTTAAATATGGCGATTCTCA  
ATTAAGCCCTACTGTTGAGCGTTGGCTTTATACTGGTAAGAATTTGTATAACGC

ATATGATACTAAACAGGCTTTTTCTAGTAATTATGATTCCGGTGTTTATTCTTAT  
TTAACGCCTTATTTATCACACGGTCGGTATTTCAAACCATTAAATTTAGGTCAG  
AAGATGAAATTAACATAAAATATATTTGAAAAAGTTTTCTCGCGTCTTTGTCTT  
GCGATTGGATTGTCATCAGCATTTACATATAGTTATATAACCCAACCTAAGCCG  
GAGGTTAAAAAGGTAGTCTCTCAGACCTATGATTTTGATAAATTCATTATTGAC  
TCTTCTCAGCGTCTTAATCTAAGCTATCGCTATGTTTTCAAGGATTCTAAGGGA  
AAATTAATTAATAGCGACGATTTACAGAAGCAAGGTTATTCATCACAATATATT  
GATTTATGTAAGTGTTCATTAAAAAAGGTAATTCAAATGAAATTGTTAAATGT  
AATTAATTTTGTCTTCTGATGTTTGTTCATCATCTTCTTTGCTCAGGTAATTG  
AAATGAATAATTCGCCTCTGCGCGATTTTGTAACTTGGTATTCAAAGCAATCAG  
GCGAATCCGTTATTGTTTCTCCCGATGTAAGGTAAGTGTACTGTATATTTCAT  
CTGACGTTAAACCTGAAATCTACGCAATTTCTTTATTTCTGTTTTACGTGCTAA  
TAATTTTGATAAGGTTGGTTCAATTCCTTCCATAATTCAAGATAATCCAAA  
CAATCAGGATTATATTGATGAATTGCCATCATCTGATAATCAGGAATATGATGA  
TAATTCGCTCCTTCTGGTGGTTTCTTTGTTCCGCAAAATGATAATGTTACTCAA  
ACTTTTAAAAATTAATAACGTTCCGGGCAAGGATTTAATACGAGTTGTGCAATTG  
TTTGTAAAGTCTAATACTTCTAAATCCTCAAATGTATTATCTATTGACGGCTCT  
AATCTATTAGTTGTTAGTGCACCTAAAGATATTTTAGATAACCTTCTCAATTC  
CTTTCTACTGTTGATTTGCCAAGTACCAGATATTGATTGAGGGTTTGATAATTTG  
AGGTTTCAAGCAAGGTGATGCTTTAGATTTTTCATTTGCTGCTGGCTCTCAGCGTG  
GCACTGTTGAGGCGGTGTTAATACTGACCGCCTCACCTCTGTTTTATCTTCTG  
CTGGTGGTTTCGTTCCGTATTTTTAATGGCGATGTTTTAGGGCTATCAGTTCCGCG  
CATTAAAGACTAATAGCCATTCAAAAATATTGTCTGTGCCACGTATTCTTACGC  
TTTCAGGTCAGAAGGGTTCTATCTCTGTTGGCCAGAATGTCCCTTTTATTACTG  
GTCGTGTGACTGGTGAATCTGCCAATGTAAATAATCCATTTCAAGACGATTGAGC  
GTCAAAATGTAGGTATTTCCATGAGCGTTTTTCTGTTGCAATGGCTGGCGGTA  
ATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCAGG  
CAAGTGATGTTATTACTAATCAAGAAGTATTGCTACAACGGTTAATTTGCGTG  
ATGGACAGACTCTTTTACTCGGTGGCCTCACTGATTATAAAAAACACTTCTCAAG  
ATTCTGGCGTACCGTTCTGTCTAAAATCCCTTTAATCGGCCTCTGTTTAGCTC  
CCGCTCTGATTCCAACGAGGAAAGCACGTTATACGTGCTCGTCAAAGCAACCA  
TAGTACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCG  
CAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTT  
CCCTTCTTTCTCGCCACGTTCCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGG  
GCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACT  
TGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCG  
CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGG  
AACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCC  
GATTTTCGGAACCAACCATCAAAACAGGATTTTCGCCTGCTGGGGCAAAACAGCGT  
GGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGT  
TGCCCGTCTCGCTGGTGAAAAGAAAAACCACCTGGCGCCCAATACGCAAACC  
GCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCC  
CGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTC  
ATTAGGCACCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAA  
TTGTGAGCGGATAACAATTTACACAGGAAACAGC

gaattcTGAAGCCTGCTTTTTTATACTAACTTGAGCGTCTAGAGTTAATAACAAGG  
AGACAGTATCGATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGT  
TTCgctagcgtagctcagGCCGGCcAAGCTTccctggtcaccgtcagcagtggtggcggaCACCCAGAAA  
CGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTAC  
ATCGAACTGGATCTCAACAGCGGTAAAGATCCTTGAGAGTTTTCGCCCCGAAGA  
ACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC  
CCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCGCATACACTATTCTCAGA  
ATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATG  
ACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGC  
CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGC  
ACAATATGGGGGATCATGTA.AACTCGCCTTGATCGTTGGGAACCGGAGCTGAAT  
GAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAA  
CAACGTTGCGCAA.AACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAAC  
AATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCG  
GCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGG  
TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTA  
GTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGAT  
CGCTGAGATAGGTGCCTCACTGATT.AAGCATTGGTA.AgtcgactaataaaAGCCCGCCT  
AATCAGCGGGCTTTTTTTTTctcgggCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC  
CCCCCTGACGAGCATCACA.AAATCGACGCTCAAGTCAGAGGTGGCGAAACCC  
GACAGGACTATAAAGATAACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCT  
CTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG  
AAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGT  
CGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCCGACCGCT  
GCGCCTTATCCGGTAACT.ATCGTCTTGAGTCCAACCCGGTAAGACACGACTTAT  
CGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGC  
GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGAC  
AGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGG  
TAGCTCTTGATCCGGCAA.ACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTG  
CAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAactGTTACACA.ACTCTTG  
TAGCCGATTAAATAAAGCGTGCAACATGGAAGCCATCACA.AACTGCATGATGAA  
CCTGTACTGAGAGCGGCAATAACAGTCTATCCCCTTCTTGCTGATATTTTGCCA  
TTGTTATAATGGGTGCA.AAATAATCGGTAAAATTAGCAACATTTAAATTAAAG  
CTATCAAAATTAACCCAAGGTAAATGCTGAAATATTTAAATGATTTTCTGGTGTT  
ACCCCTTGAGGAAAT.AACTTGGTATCACTTTTATAACGTTCCATTACCGATAAA  
TAATTCACCATAAATTGATCAATATCGGATGAGTATGGGCAACTCAGTGCTGA  
AAATGTCTCTGTTTCTTGATGGAATACGGTGAATTGTGGaTcaACTGAATCCCAT  
ACGATCAATTCATCATCTTTTATCGCCATTCTCAACTCATCAAATTGATTACG  
GCCTGAGCAATCAGATAGATCATTACCGGATAAAACTTATACGCTGAATCATC  
CAATGACTTTTTTAAACGTCGTGATATCAATTTTGCTTGTTAAGCTAAAACCACA  
TGGTAAACGATGCCGATAAAACTCAAATGCTCACGGCGAACCCAATTTTTTA

FIG. 5-1

CATCAAATTTTGTATAGTTCATAATCGATCCCTTCTGAATTTTATTTACTAAGA  
ACCTATCCCAAAGGATTTTATTCCAGACAATGATGCACAGGCAAGA

gaattcTGAAGCCTGCTTTTTTATACTAACTTGAGCGTCTAGAGTTAATAACAAGG  
AGACAGTATCGATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGT  
TTCgctagcgtagctcagGCCGGCTGGTGGCGGTGGCTCCcAAGCTTccctgggtcaccgtcagcagt  
ggtggcggaCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGG  
TGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGA  
GTTTTCGCCCCGAAGAACGTTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTAT  
GTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGC  
ATACACTATTCTCAGAATGACTTGTTGAGTACTCACCAGTCACAGAAAAGCA  
TCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGA  
GTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG  
CTAACCGCTTTTTTGCACAATATGGGGGATCATGTAACCTCGCCTTGATCGTTGG  
GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGC  
CTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACCTGGCGAACTACTTACT  
CTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGG  
ACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGG  
AGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTA  
AGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGAT  
GAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTA  
AgtcgactaataaAGCCCGCCTAATCAGCGGGCTTTTTTTTctcgggCCGCGTTGCTGGCG  
TTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGT  
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGG  
AAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTC  
CGCCTTTCTCCCTTCGGGAAGCGTGCGCTTTTCTCATAGCTCACGCTGTAGGTA  
TCTCAGTTCGGTGTAGGTCTGTTCTGCTCCAAGCTGGGCTGTGTGCACGAACCCCC  
CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCC  
GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCA  
GAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC  
GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTAC  
CTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTA  
GCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCT  
CAactGTTACACAACCTCTTGTAGCCGATTAATAAAGCGTGCAACATGGAAGCCA  
TCACAAACTGCATGATGAACCTGTACTGAGAGCGGCAATAACAGTCTATCCCC  
TTCTTGCTGATATTTTGCCATTGTTATAATGGGTGCAAAATAATCGGTAAAATT  
AGCAACATTTAAATTAAGCTATCAAAATTAACCCAAGGTAATGCTGAAATAT  
TTAAATGATTTTCTGGTGTACCCCTTGAGGAAATAACTTGGTATCACTTTTATA  
ACGTTCCATTACCGATAAATAATTCACCATAAATTGATCAATATCGGATGAGTA  
TGGGCAACTCAGTGCTGAAAATGTCTCTGTTTCTTGATGGAATACGGTGAATTG  
TGGaTCaACTGAATCCCATACGATCAATTCATCATCTTTTATCGCCATTCTCAAC  
TCATCAAATTGATTCACGGCCTGAGCAATCAGATAGATCATTACCGGATAAAA  
CTTATACGCTGAATCATCCAATGACTTTTTTAACGTCGTGATATCAATTTTGCTT  
GTTAAGCTAAAACCACATGGTAAACGATGCCGATAAACTCAAATGCTCACG  
GCGAACCCAATTTTTTACATCAAATTTTGTATAGTTTATAATCGATCCCTTCTG  
AATTTTTATTACTAAGAACCTATCCCAAAGGATTTTATTCCAGACAATGATG  
CACAGGCAAGA

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/18207

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/72; C12P 21/02

US CL : 435/320.1, 69.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
BRS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,658,727 A (BARBAS et al.) 19 August 1997, entire document.	1-8, 11-24
Y	US 5,427,908 A (DOWER et al.) 27 June 1995, entire document.	5, 14



Further documents are listed in the continuation of Box C.



See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
--------------------------------------	---	--------------------------	--

Date of the actual completion of the international search

17 NOVEMBER 1999

Date of mailing of the international search report

09 DEC 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JAMES KETTER

Telephone No. (703) 308-0196